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Comparative Analysis of Nucleotide Sequence and Codon Usage of Arylphorin Gene Cloned from Four Silk-Producing Insects and Their Molecular Phylogenetics

Jae-Sam Hwang, Jin-Sung Lee*, Tae-Won Goo, Sang-Mong Lee†, O-Yu Kwon***, and Ho-Rak Kim**

National Sericulture & Entomology Research Institute, R.D.A., Suwon 441-100, Korea
**Genome Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), KIST, Yusong, Taejon 305-600, Korea*
***Dept. Sericulture & Science, Miryang National Univ., Miryang, Kyung-nam 627-130 Korea*
****Dept. of Anatomy, Coll. of Medicine, Chungnam National. Univ., Taejon 301-131, Korea*

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Abstract To determine phylogenetic relatedness of four silk-producing silkmoths (*B. mori. B. mandarina, A. yamamai and A. pernyi*), internal coding region of arylphorin which is a storage protein in hemolymph protein of insects were amplified by polymerase chain reaction and then sequenced and compared each other. The nucleotide composition was biased toward adenine and thymine (59% A+T) and a strong bias for use of C in the third position of codons was found for Phe and Tyr. Together TTC (Phe) and TAC (Tyr) account for about 16.8% (10 for TTC and 8 for TAC) of all codon usage. The nucleotide similarity of arylphorin gene from *B. mori* showed 99%, 98% and 97% homology with those of *B. mandarina*, *A. yamamai* and *A. pernyi*, respectively. Also, the nucleotide sequence of arylphorin gene from *B. mandarina* showed 98% and 97% homology with those of *A. yamamai* and *A. pernyi*, respectively. Between *A. yamamai* and *A. pernyi*, the sequence homology was 97%. The deduced amino acid sequences in *B. mori*, *B. mandarina* and *A. yamamai* showed almost 99% homology. Although the arylphorin gene provided insufficient variability among the four insect species, A UPGMA tree is generated that supported the monophyly of silk-producing insects, with *M. sexta* placed basal to it. It is suggest that silk-producing insects have a close relationship and a homogeneous genetic background from comparison with those of other insects.

Key words: Arylphorin gene, Silk-producing insects, Codon usage, Phylogenetics

Introduction

Silkmoths are holometabolous insects belonging to either the family of Bombycidae or Saturniidae, both of which belong to the superfamily of Bombycoidea. Although the two families are similar in some respect, they also have many differences in external and internal features. Molecular taxonomic and phylogenetic information for the silkmoths should reinforce conventional information and provide efficient ways for preservation and utilization of genetic resources silkmoths. Arylphorin is tyrosine and phenylalanine-rich storage protein found in the hemolymph of various insects. It is presumed that arylphorin is amino acid reservoir for cuticle formation during molting and metamorphosis [5,8,13]. Arylphorin genes have been isolated from various species such as *Drosophila melanogaster*, *Manduca sexta* and *Bombyx mori*[2,8,12]. Arylphorin represents important model protein because their genes are highly expressed and are developmentally regulated according to the developmental stage. The genes for the three arylphorin subunits from *Drosophila melanogaster* and for the arylphorin from *Saccrophage peregrina* have been cloned and some partial sequence data reported. However, to date, no arylphorin gene has been reported from domestic and wild type silkmoths (*B. mandarina, A. pernyi and A. yamamai*).

In the present study, to get a genetic information and a molecular characteristic for Bombycoidea superfamily, the portion of arylphorin genes between Bombycidae and Saturniidae were partially amplified by PCR and nucleotide sequences and codon usage were compared. In addition, the phylogenetic relationship was compared with silkmoths and some insect species

Materials and Methods

Genomic DNA was purified from the posterior silkgland of *B. mori, B. mandarina, A. yamamai* and *A. perny*, respectively. About 0.5 g of posterior silkgland was dissected from

†Corresponding author
Phone: 82-331-291-8281
E-mail: SERILSM@arang.miryang ac.kr

the larvae on 3rd day of the final instar. The frozen silkgland by liquid nitrogen was homogenized in a buffer consisted of 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% SDS and proteinase K and incubated at 37℃ for 30 min. After extraction with phenol three times and phenol and chloroform (1:1) two times, the supernatant was precipitated with two volume of ethanol. The DNA pellet was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) containing RNase (10 ug/ml).

Primers for PCR were designed on the basis of highly conserved sequences of *B. mori* and *Manduca sexta* [2,6]. The partial region of arylphorin gene was confirmed using PCR with the primers (AP1; 5'-TTCCACTCGCACTTACCGTTCTGGTGG, AP2; 5'-CCATACAGATCAGCATTATCTTGCCA). DNA fragments were amplified in a total volume of 20$\mu\ell$ using 40 ng of genomic DNA and 0.25 unit of *Taq* polymerase. PCR condition was one cycle at 96℃, 3min and following 35 cycles at 96℃ for 1min, 50℃ for 1min and 72℃ for 1min.

The PCR products were separated on 1% agarose gel and the expected bands size were recovered using Gene Clean II kit (Bio 101, USA). The eluted fragments were directly ligated with pGem-T easy vector (Promega, USA) and transformed into *E. coli* JM 109. Fluorescent cycle sequencing was performed using 300-500 ng DNA as template in 20$\mu\ell$ reaction volume, and DNA sequences were carried out ABI PRISM 377 DNA sequencer (Perkin Elmer, USA). The multialignments were analyzed using CLUSTAL W[14] and their genetic distance and phylogenetics tree were constructed using UPGMA clustering method by MEGA program. These two analyses were trimmed for maximizing data set of the nucleotide sequence.

Results and Discussion

From four silkmoths (*B. mori*, *B. mandarina*, *A. yamamai* and *A. pernyi*), *Partial fragment of* arylphorin gene was amplified by PCR using forward and reverse primer which were determined on the basis of the arylphorin sequences from *B. mori* and *Manduca sexta* [2,16]. The PCR conditions used in this experiment shown in the 'Materials and Methods' were performed on the PCR design program of DNASTAR program. As can be seen in Fig.1. the PCR products of 0.4kb were successfully amplified from *B. mori*, *B. mandarina*, *A. yamamai* and *A. pernyi*, respectively. The fragments were eluted, cloned into the T-overhang vecter (pGem-T easy vector) and sequenced.

The base composition of four silkmoths and A+T ratio were almost identical with 29% A, 18% G, 29% T and 22% C, and 59% A+T. Therefore, the base composition of arylphorin genes from four silkmoths is heavily biased toward adenine and thymine.

Table 1 presents the codon usage and its comparisons of deduced amino acid of arylphorin gene from four

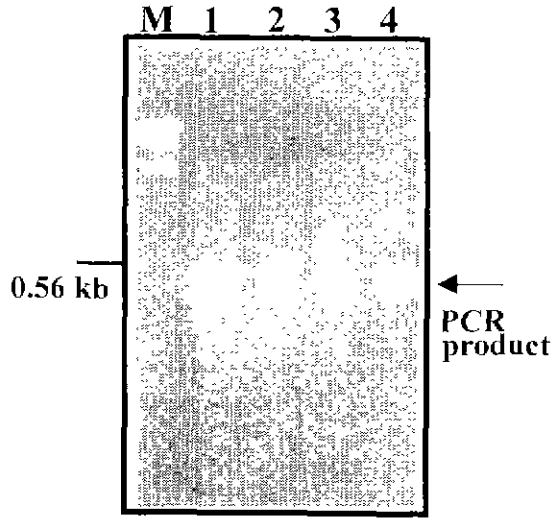

Fig. 1. PCR product amplified by AP1 (5'-TTCCACTCGCACTTACCGTTCTGGTGG) and AP2 (5'-CCATACAGATCAGCATTATCTTGCCA) primers. The lanes are M: molecular marker of λ /*Hin*dIII, lane 1: *B. mori*, lane 2: *B. mandarina*, lane 3: *A. yamamai*, lane 4: *A. pernyi*.

silkmoth. In the apolipophorin-III from *M. sexta* and *Locusta migratoria* a bias toward the use of C or G in third position of codon was noted. In arylphorin genes a strong bias for use of C in the third position of codons is found for Phe and Tyr. Together TTC (Phe) and TAC (Tyr) account for about 16.8 % (10 for TTC and 8 for TAC) of all codon usage, which means that the corresponding tRNAs must be abundant in order to support the high rates of arylphorin biosynthesis seen in the last stage.

Nucleotide sequence data were aligned using CLUSTAL W program as shown in Fig. 2. The sequence identity of Arylphorin gene between the Bombycidae (*B. mori* and *B. mandarina*) and Saturniidae (*A. yamamai* and *A. pernyi*) was analyzed. As a result, the nucleotide sequence of arylphorin gene from *B. mori* showed 99%, 98% and 97% sequence homology with those of *B. mandarina*, *A. yamamai* and *A. pernyi*, respectively. And also, the nucleotide sequence of arylphorin gene from *B. mandarina* showed 98% and 97% homology with those of *A. yamamai* and *A. pernyi*, respectively. Between *A. yamamai* and *A. pernyi*, the sequence homology was 97%. The deduced amino acid sequences in *B. mori*, *B. mandarina* and *A. yamamai* showed almost 99% homology (Fig. 3). However, from the comparison with other reported arylphorin which is *Aedes aegypti* (U86080), *Actias artemis* (D44482) *Caligula boisduvalii* (D44488), *Hyalophora cecropia* (AF032396) and *Manduca sexta* (M28395), nucleotide similarities showed a high match at 5' region, and a low match at 3' region.

An evolutionary tree showing a possible relationship between four silkworms and above the five insects based

Table 1. Comparison of amino acid composition and codon usage of arylphorin gene from four silk-producing silkmoths

Amino acid	*B. mandarina*	*B, mori*	*A. yamamai*	*A. pernyi*
Ala	4	4	4	4
Arg	7	7	7	6
Asn	4	4	4	4
Asp	3	3	3	3
Cys	1	1	1	1
Gln	5	5	5	6
Glu	10	10	9	10
Gly	5	5	6	5
His	4	4	4	4
Ile	5	5	6	5
Leu	13	13	13	13
Lys	8	8	8	8
Met	1	1	1	1
Phe (TTC)	10	13	13	12
Phe (TTT)	3	3	3	3
Phe (Total)	13	10	10	10
Pro	6	6	6	6
Ser	6	6	7	6
Thr	8	8	7	8
Trp	3	3	3	3
Tyr (TAC)	10	10	8	11
Tyr (TAT)	4	4	5	4
Tyr (Total)	14	14	13	15
Val	5	5	5	5

on the nucleotide sequence data presented in Fig. 4. This tree was derived from the aligned sequences and the distance scores given in Table 2, and a bootstrap analysis with 1000 replication was performed on the entire data set. This tree is consistent with the phylogentic relationships of the species from which deduced amino acid sequences data were obtained. A UPGMA tree is generated that supported the monophyly of silk-producing insects, with *M. sexta* placed basal to it. Among the four silk-producing silkmoths, genetic relationship of *B. mori* and *B. mandarina* is closer than that of *A. yamamai* and *A. pernyi*.

Although the two families of Bombycidae and Saturniidae are similar in some respects of genetics, they have many differences in the morphologic features and their behavior except for producing silk. So far, many studies have been examined for the relationship between Bombycidae and Saturniidae. *B. mori* and *B. mandarina* were crossed hereditarily and the resulting hybrid progeny shows normal fertility [1,3]. From studies for fibroin gene, ribosomal gene and immunological properties of aryphorin between *B. mori* and *B. mandarina*, the obtained data suggest that they are a close homology in genetic background [6,7,16]. *A. yamamai* and *A. pernyi* are economically important wild silkmoths. Although their chromosome numbers are quite different (yamamai=31, pernyi=49), they were clossed to produce F_1 progeny [4,10]. However, the progeny is due to incomplete oogenesis and other abnomalities [9]. Kirimura showed that fibroin of *A yamamai* and *A pernyi* are clearly similar than those of B. mori and B. mandarina [9].

In this study, we have suggested based on partial sequences of arylphorin gene that there might existed a more closer genetic relationship between *B. mori* and *B. mandarina* than that of *A. yamamai* and *A. pernyi*. Although this report is not a full evidence to elucidate that four silkmoths are a cluster as monophyly and *B. mandarina* which inhabits nature, it is possible ancestor of the domesticated *B. mori*. This is one of the data to clue the relationship of four silk-producing silkmoths on the arylphorin gene level. Our results eventually indicate that gene encoding arylphorin is distributed on the chromosomes as a well conserved gene which play as a systematic tool for molecular evolution.

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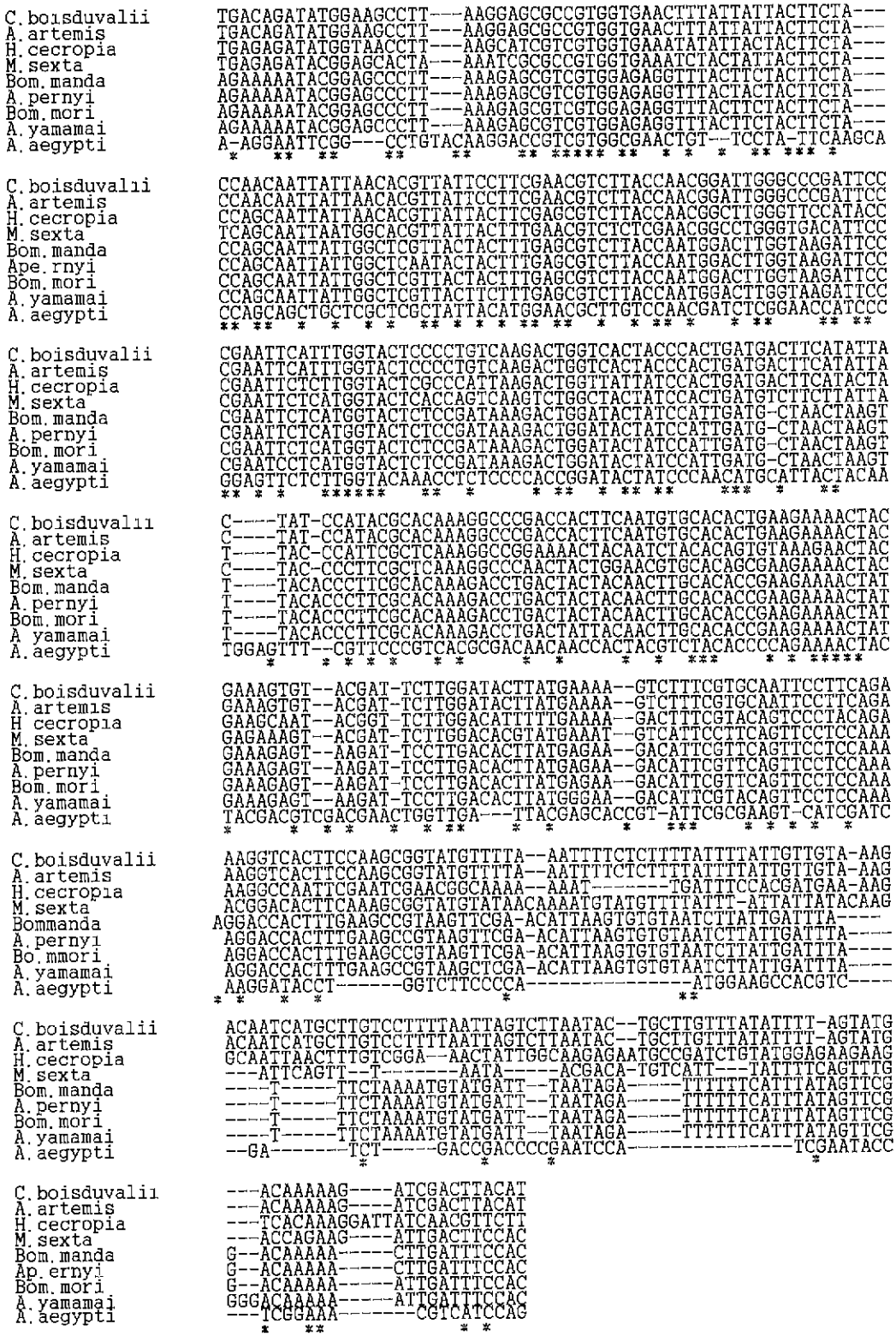

Fig. 2. Muti-alignment of PCR amplified nucleotide sequence of the arylphorin genes. Asterisks and hyphens indicate nucleotide common and gap to all species. [Bom. mori: *Bombyx mori*, Bom. manda: *Bombyx mandarina*, A yamamai: *Antheraea. yamamai*, A.pernyi: *Antheraea pernyi*, A. aegypti. *Aedes aegypti* (U86080), A.artemis: *Actias artemis* (D44482), C.boisduvalii: *Caligula boisduvalii* (D44488), H. ceropia: *Hyalophora cecropia* (AF032396), M. sexta: *Manduca sexta* (M28395)]

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Ape     FHSHLPFWWTSEKYGALKERRGEVY[ ]YFYQQLLA[ ]YYFERLTNGLGKIPEFSWYSPIKTG
Bma     FHSHLPFWWTSEKYGALKERRGEVYFYFYQQLLARYYFERLTNGLGKIPEFSWYSPIKTG
Bmo     FHSHLPFWWTSEKYGALKERRGEVYFYFYQQLLARYYFERLTNGLGKIPEFSWYSPIKTG
Aya     FHSHLPFWW[ ]SEKYGALKERRGEVYFYFYQQLLARY[ ]FERLTNGLGKIPE[ ]SWYSPIKTG
        ********* ************** ******** * ************ *********

Ape     YYPLMLTKFTPFAQRPDYYNLHTEENYERVRFLDTYEKTFVQFLQKDHFEAVSSNIKCVI
Bma     YYPLMLTKFTPFAQRPDYYNLHTEENYERVRFLDTYEKTFVQFLQKDHFEAVSSNIKCVI
Bmo     YYPLMLTKFTPFAQRPDYYNLHTEENYERVRFLDTYEKTFVQFLQKDHFEAVSSNIKCVI
Aya     YYPLMLTKFTPFAQRPDYYNLHTEENYERVRFLDTY[ ]KTFVQFLQKDHFEAVSSNIKCVI
        ************************************ ************************

Ape     LLIYF
Bma     LLIYF
Bmo     LLIYF
Aya     LLIYF
        *****
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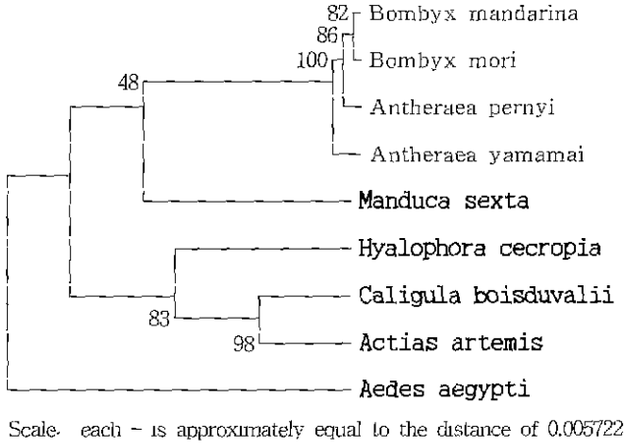
Fig. 3. Multi-alignment of amino acid sequences deduced of arylphorin gene from four silk-producing insects (Bmo; *B. mori*, Bma; *B. mandarina*, Aya; *A. yamamai*, Ape; *A. pernyi*)

Table 2. Genetic distnact of arylphorin genes among nine insect species based on nucleotide sequences

	C. boi	A. art	H. cec	B. man	A. per	B. mor	A. yam	M. sex	A.aeg
A. art	0.2322								
H. cec	0.3126	0.3331							
B. man	0.4326	0.4495	0.5039						
A. per	0.4379	0.4550	0.5100	0.0081					
B. mor	0.4281	0.4448	0.5047	0.0027	0.0108				
A. yam	0.4517	0.4719	0.5267	0.1090	0.0273	0.0163			
M. sex	0.3373	0.3846	0.4376	0.3676	0.3724	0.3633	0.3856		
A. aeg	0.8425	0.8089	0.7744	0.8693	0.8859	0.8700	0.9104	0.8720	



Scale- each – is approximately equal to the distance of 0.005722

Fig. 4. Phylogenetic tree showing the evolutionary relationship of silkmoth arylphorin gene with reported insects arylphorin gene. [Amo: *B. mori*, Ama: *B. mandarina*, Aya: *A. yamamai*, Ayp: *A. pernyi*. *Aedes aegypti* (U86080), *Actias artemis* (D44482) *Caligula boisduvalii* (D44488), *Hyalophora cecropia* (AF032396) and *Manduca sexta* (M28395)]

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